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Effect of inserting charged peptide at NH_2-terminal on N-type inactivation of Kv1.4 channel

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ABSTRACT

Rapid inactivation of voltage-gated potassium channel plays an important role in shaping the electrical signaling in neurons and other excitable cells. N-type ("ball and chain") inactivation, as the most extensively studied inactivation model, is assumed to be the inactivation mechanism of Kv1.4 channel. The inactivation ball inactivates the channel by interacting with the hydrophobic wall of inner pore and occluding it. Recently, we have proved that the electrostatic interaction between two charged segments in the NH_2-termainal plays an important role through promoting the inactivation process of the Kv1.4 channel. This study investigates the effect of inserting negatively or positively charged short peptides at NH_2-terminal on the inactivation of Kv1.4 channel. The results that inserting negatively-charged peptide (either myc or D-peptide) at different sites of NH_2-terminal, deceleraes inactivation process of Kv1.4 channel to a different extent with inserting site changing and that the mutant Kv1.4-D50 exhibits a more slower inactivation rate than Kv1.4-K50 further identified the role of electrostatic interactions in the "ball and chain" inactivation mechanism.

1. Introduction

A-type current of potassium ion channel, characterized by its rapid inactivation after activation, plays an important role in shaping action potentials and modulating the firing frequency of neurons in the central nervous system [1]. The most extensively studied inactivation mechanism of A-type current is N-type inactivation which is also called "ball and chain" model. In this inactivation model, an amino terminal inactivation ball tethered by its carboxyl hydrophilic chain acts as an open channel blocker and occludes the channel mouth through hydrophobic interactions shortly after its opening [2,3]. The "ball and chain" inactivation model was first proposed by Armstrong & Bezanilla in studying the inactivation mechanism of the voltage-gated Na^+ channel [4–6]. Subsequently, the *Drosophila Shaker* K^+ channel (ShB) and some other voltage-gated potassium channels, like Kv1.4, Kv3.3, Kv3.4 and Kv4.2, were also identified to exhibit the "ball and chain" inactivation mechanism [2,3,7–10]. The inactivation ball plays a critical role in the "ball and chain" inactivation model. The rapid inactivation can be abolished by deletion of the inactivation ball at the NH_2-terminus whereas application of a synthetic peptide derived from the inactivation domain from cytoplasmic side can restore or induce rapid inactivation currents in NH_2-terminal deleting mutants or other non-inactivating channels [2,3,11]. The chain links the NH_2-terminal ball to the transmembrane body and determines the diffusing rate of the inactivation ball towards its binding site [2]. For ShB channel, the first 20 amino acids constitute the inactivation ball and the subsequent 60 residues play a role as the hydrophilic chain [2,3]. For Kv1.4 channel, the first 38 amino acids are often thought to form the inactivation ball [7]. However, some other studies proposed that Kv1.4 channel includes two inactivation balls and residues 40–68 (or residues 40–50) constitute another inactivation ball besides the ball formed by residues 1–38 [12,13]. The inactivation chain of Kv1.4 has not been identified clearly so far.

The inactivation ball of either ShB or Kv1.4 comprises an NH_2-terminal hydrophobic region and a COOH-terminal hydrophilic region. The hydrophobic region plays a role in inactivation by interacting with the hydrophobic wall of the inner pore [14–16]. The hydrophilic region carrying several net positive charges accelerates the inactivation process by electrostatic interactions [2,7,15,17]. We have demonstrated that the positively charged inactivation ball of Kv1.4 channel interacts with the negatively charged T1-S1 linker region by electrostatic attractions in our previous study [18]. In this study, we further investigate the effect of electrostatic attractions or repulsions induced by negatively- or positively-charged short peptides inserted at different positions of amino terminal on inactivation process of Kv1.4 channel.

2. Materials and methods

2.1. Molecular biology

The sequence information of rat Kv1.4 used in this study can be obtained from GenBank accession number 012971 [19]. The first 70 amino acids at the NH_2-terminal for wild type and mutant Kv1.4 channels

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are listed in Fig. 1. Negatively-charged peptide (myc-peptide or D-peptide) or positively-charged peptide (K-peptide) was inserted behind the amino acid which is marked at the corresponding site. The amino acid sequence of c-myc tag, D-peptide and K-peptide are listed at the bottom of Fig. 1. All of the mutants were generated by overlap PCR. In the first round PCR, 4 primers were used to obtain two fragments (fragment I and fragment II) both of which include an overlapping amino acid sequence corresponding to the inserting peptide. The two fragments were ligated together in the second round PCR. Then the ligated fragment which includes the intact inserting peptide was cut with *Eco*RI and *Bam*HI at the 5' and 3' end respectively and subcloned into a *pcDNA3* vector cut with the same restriction enzymes. The plasmids containing the mutation genes were finally transformed into DH5α-competent cells. After enzymatic or PCR identification, all of the mutants were confirmed by sequencing to ensure the absence of errors.

2.2. Cell culture

CHO-K1 cells were grown in Ham's F-12 nutrient mixture (Invitrogen, Co. Grand Island, N.Y.) supplemented with 10% fetal bovine serum, in a humidified 37 °C incubator (5% CO_2). The cells were passaged twice weekly through exposure to 0.05% trypsin, diluted in 0.5 mM EDTA in PBS. For gene transfection, the cells were transferred to poly-L-lysine coated glass coverslips. After cell density reached 50-–70% confluence, rKv1.4WT or its mutant gene was transiently co-expressed with pEGFP (Clontech, Palo Alto, CA) using LipofectAMINE Plus(TM) reagent (Invitrogen). Cells showing GFP fluorescence were chosen after 24 hours of transfection for use in electrophysiological experiments.

2.3. Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature (~22°C) with an EPC-10 amplifier (HEKA, Lambrecht, Germany). Pipettes were pulled from borosilicate glass and had a resistance of between 2 and 4 MΩ. The pipette solution was (in mM): 140 KCl, 10 EGTA, 1 $CaCl_2$, 2 Mg-ATP and 10 HEPES (pH 7.2, with KOH). The extracellular solution was Hanks' balanced salts solution (HBSS, Sigma; in mM): 1.3 $CaCl_2$, 0.8 $MgSO_4$, 5.4 KCl, 0.4 KH_2PO_4, 136.9 NaCl, 0.3 Na_2HPO_4, 10 D-glucose and 4.2 $NaHCO_3$. Currents were recorded with a sampling frequency of 5.0 kHz. Capacitative and leak currents were subtracted, and series resistance compensation (80%) was used for currents exceeding 2 nA. Holding potential was maintained at −90 mV. To obtain activation curves, the currents were evoked by step depolarization to test potentials between −90 and +70 mV for 500 ms in 20 mV increments. Steady-state inactivation curves were obtained using a standard two-step protocol, which involved a pre-pulse of 1 s or 2 s (for Kv1.4-D50) at potentials between v90 and +50 mV (10-mV increments) and a test pulse of +30 mV to determine the fraction of channels inactivated during the pre-pulse.

2.4. Data acquisition and analysis

Data were acquired with the Pulse/PulseFit program (HEKA, Lambrecht, Germany) and further analyzed using Origin 7.0 (OriginLab, Northampton, MA). The inactivation time constant (τ_{inact}) was obtained by using a single exponential function fitted to the decaying curve of current trace recorded at +70 mV. Boltamann equation $G/Gmax = 1/[1+\exp(V_{1/2}-V)/k]$ and the modified Boltzmann equation $I/Imax = 1/[1+\exp(V-V_{1/2})/k]$ were used to fit to the voltage-dependence of activation and inactivation curves respectively. Data were shown as means ± S.E.M. Statistical analysis was carried out by one-way ANOVA using a Bonferroni test. The criterion for a significant difference was $p<0.05$.

3. Results

3.1. Effect of inserting c-myc tag at different sites of NH_2-terminal of Kv1.4

To investigate the effect of inserting a negatively-charged peptide on inactivation of Kv1.4, we constructed 3 mutants which had a c-myc (EQKLISEEDL) tag inserted behind 19Y, 39A and 50A respectively. C-myc tag is a 10-amino-acid short peptide and has 3 net negative charges. The macroscopic current traces of Kv1.4WT and the three mutants are shown in Fig. 2A−D. Fig. 2E shows the normalized and superimposed current traces at +70 mV for the 4 constructs. Of all

Fig. 1. Schematic representations of the wild type and mutant Kv1.4 channels. The amino acid sequence of 1–70 of wild type Kv1.4 is shown on the top and the 7 inserting mutants with inserting peptide marked are shown below. The amino acid sequences of the 3 inserting peptide are listed at the bottom. myc-peptide: EQKLISEEDL; D-peptide: SGDEDSG; K-peptide: SGKRKSG.

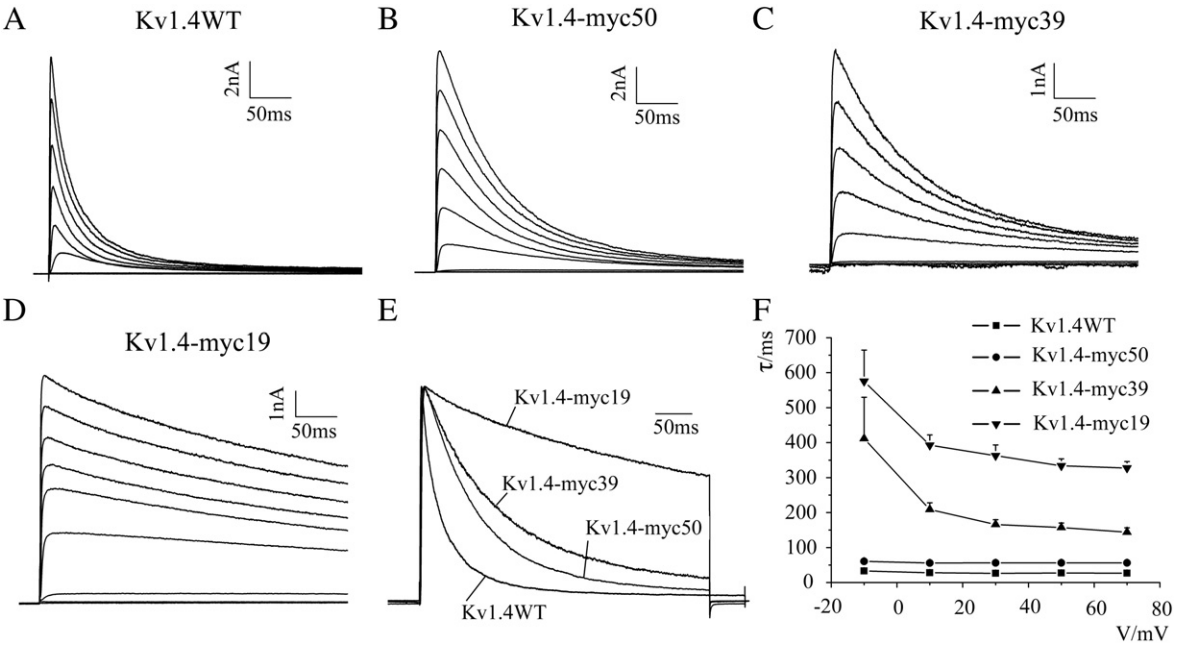

Fig. 2. Effect of inserting myc-peptide on inactivation of Kv1.4 channel. (A-D) Representative whole-cell current traces for Kv1.4WT, Kv1.4-myc50, Kv1.4-myc39 and Kv1.4-myc19 respectively, which were transiently expressed in CHO-K1 cells. (E) The normalized and superimposed current traces for the 3 myc-peptide inserting mutants and Kv1.4WT at +70 mV membrane potential. (F) Comparison of inactivation time constants (τ_{inact}) for Kv1.4WT and the mutants at different membrane voltage (from –10 mV to +70 mV). The currents were evoked by step depolarization to test potentials between –90 and +70 mV for 500 ms in 20 mV increments. Inactivation time constants (τ_{inact}) were obtained by using a single exponential function fitted to the decaying period of current traces.

the constructs, Kv1.4WT has the most rapid inactivation rate and the inactivation rates of Kv1.4-myc50, Kv1.4-myc39 and Kv1.4-myc19 become slower and slower. The inactivation time constants of Kv1.4WT, Kv1.4-myc50, Kv1.4-myc39 and Kv1.4-myc19 at +70 mV are 26.8 ± 1.4 ms (n = 6), 56.0 ± 3.8 ms (n = 9), 143.8 ± 12.5 ms (n = 5) and 327.4 ± 18.5 ms (n = 5) respectively. This change does not show apparently voltage-dependence from −10 mV to +70 mV at which the currents are activated for all the constructs (Fig. 2F). The decelerating effect by inserting c-myc tag at amino terminal on inactivation rate of Kv1.4 channel indicates that this negatively charged peptide influences the inactivation process of Kv1.4 channel, and its influence become greater with the inserting site moving to the extreme NH_2-terminal. Considering the electrostatic interaction between inactivation domain and T1-S1 linker which was identified in our previous study [18], the decelerating effect caused by inserting myc-peptide could be attributed to the electrostatic repulsion between negatively-charged myc-peptide and T1-S1 linker.

3.2. Effect of inserting another negatively charged peptide on inactivation of Kv1.4 channel

Next, we inserted another peptide (D-peptide: SGDEDSG), which has 3 negatively-charged amino acids gathering in the middle, at the same position as c-myc inserting mutants. Similarly, all these mutants have a slower inactivation rate than Kv1.4WT. The inactivation time constants of Kv1.4-D19, Kv1.4-D39 and Kv1.4-D50 are 582.6 ± 71.5 ms (n = 4), 380.4 ± 14.5 ms (n = 5) and 216.1 ± 20.6 ms (n = 5) respectively. Like myc-tag inserting mutants, the inactivation rates of these mutants also become slower and slower with the inserting position moving towards the extreme amino terminus (Fig. 3A−C). Fig. 3D to F show the normalized current traces at +70 mV of the 3 pairs of mutants (Kv1.4-D50 vs Kv1.4-myc50, Kv1.4-D39 vs Kv1.4-myc39 and Kv1.4-D19 vs Kv1.4-myc19). The inactivation time constants of each pair of mutants are compared through bar graphs in Fig. 3G-I (216.1 ± 20.6 ms and 56.0 ± 3.8 ms for Kv1.4-D50 and Kv1.4-myc50; 380.4 ± 14.5 ms and 143.8 ± 12.5 ms for Kv1.4-D39 and Kv1.4-myc39; 582.6 ± 71.5 ms and 327.4 ± 18.5 ms for Kv1.4-D19 and Kv1.4-myc19). Despite the same net charges, the D-peptide inserting mutant inactivates more slowly than the myc-peptide inserting mutant with the same inserting site. These results show that the negatively charged D-peptide is more effective in decelerating the inactivation rate of Kv1.4 channel than the myc-peptide. Comparing with D-peptide which has 3 consecutive negatively-charged amino acids in the middle, myc-peptide has a more sparse distribution of charges and even has a positively-charged residue K at the amino terminal. Perhaps the different distribution and the positive charge of K make the decelerating effect of myc-peptide weaker than that of D-peptide.

3.3. Effect of inserting a positively charged peptide after 50A on inactivation of Kv1.4

For comparison, we chose the position 50A after which a positively charged peptide (K-peptide: SGKRKSG) was inserted. Fig. 4A and B show the macroscopic current traces of Kv1.4WT and Kv1.4-K50 respectively. The normalized and superimposed current traces at +70 mV of Kv1.4WT, Kv1.4-K50 and Kv1.4-D50 are shown in Fig. 4C. The inactivation time constant of Kv1.4-K50 is 42.2 ± 4.0 ms (n = 9, Fig. 4D), which is far smaller than that of Kv1.4-D50 (216.1 ± 20.6 ms) but significantly larger than that of Kv1.4WT (26.8 ± 1.4 ms). The inserted positively-charged K-peptide does not make an effective acceleration to the inactivation process of Kv1.4 channel. These results indicate that the original positive charges of inactivation domain are just adequate for accelerating the inactivation process by electrostatic interaction with T1-S1 linker region and adding more positive charges does not play a further role but decelerates it contrarily.

3.4. The voltage-dependence of steady state inactivation

Next, we compared the voltage-dependent properties of steady-state inactivation of Kv1.4WT, Kv1.4-D50 and Kv1.4-K50. The voltage-dependence of inactivation was measured by a standard two-pulse protocol with an inactivation pre-pulse (P1) over the range −90

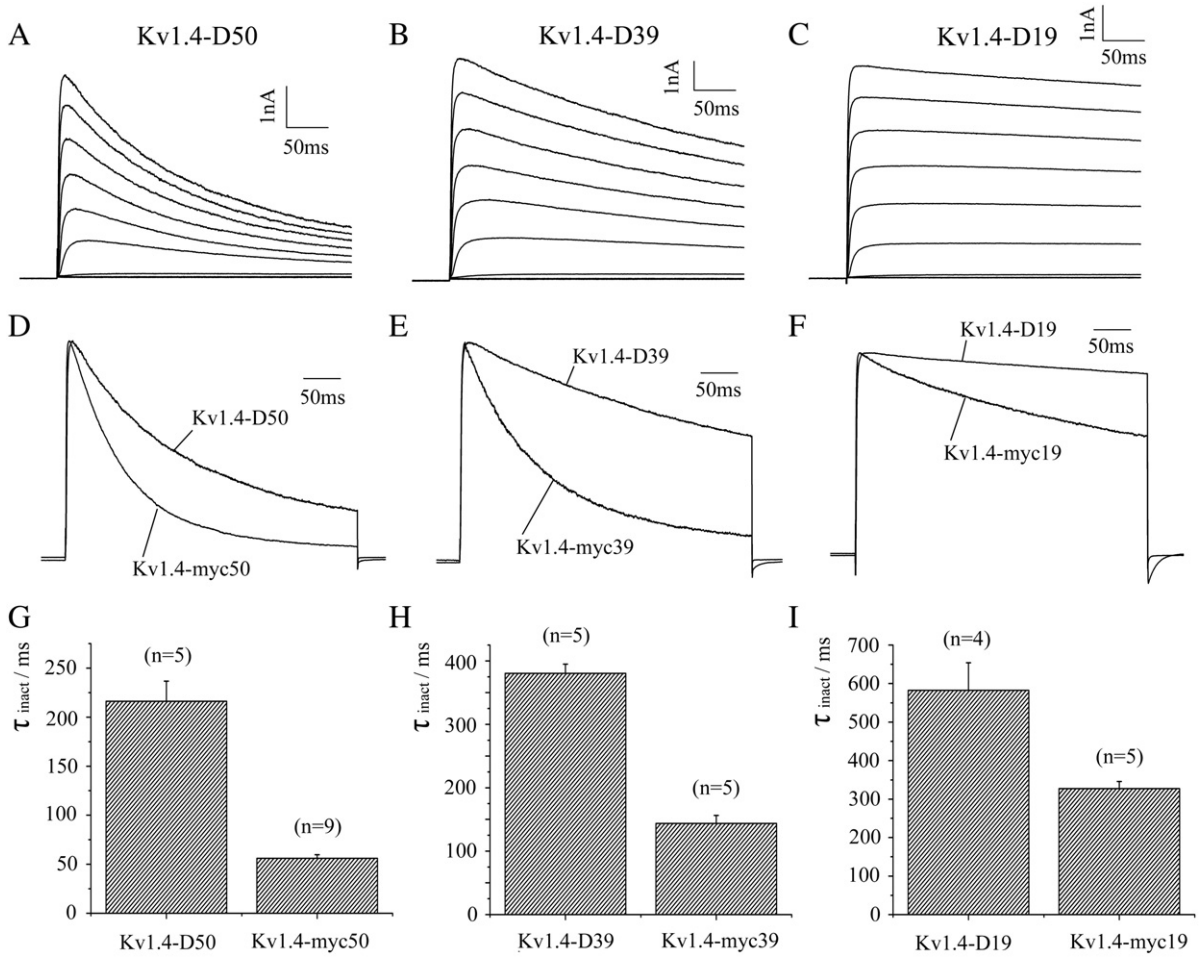

Fig. 3. Comparison of inactivation for D-peptide inserting mutants with that of myc-peptide inserting mutants. (A-C) Representative whole-cell current traces for Kv1.4-D50, Kv1.4-D39 and Kv1.4-D19 respectively. (D-F) The normalized and superimposed current traces for myc- and D- peptides inserting mutants at +70 mV with inserting sites behind residues 50, 39 and 19 respectively. (G-I) Comparison of inactivation time constants (τ_{inact}) at +70 mV for the 3 pairs of mutants. The number of cells tested is given in the parentheses.

to +50 mV followed by a test pulse (P2) to +30 mV. Fig. 5A-C show the currents of Kv1.4WT, Kv1.4-D50 and Kv1.4-K50 elicited upon the two-pulse protocol respectively. Fig. 5D shows the normalized peak currents of test pulse for the 3 constructs plotted as a function of pre pulse potentials. The half-inactivation potentials ($V_{1/2}$) of Kv1.4WT, Kv1.4-D50 and Kv1.4-K50 are −53.1 ± 0.7 mV(n = 12), −52.5 ± 0.9 mV(n = 15) and −51.2 ± 0.9 mV(n = 12) respectively and do not show significant differences, suggesting that the mutations do not affect the voltage-dependent inactivation of the channel.

3.5. The voltage-dependence of activation

The voltage-dependence of activation for Kv1.4WT, Kv1.4-D50 and Kv1.4-K50 were also examined. The fractional conductance of the constructs are plotted against the membrane voltages and fitted to the Boltzmann function (Fig. 6). The overlapping curves in Fig. 6 indicate the similar properties of voltage-dependence for the two mutants and Kv1.4WT channel. The half activation voltages for Kv1.4WT, Kv1.4-D50 and Kv1.4-K50 are −16.3 ± 0.4 mV (n = 8), −17.3 ± 1.1 mV (n = 11) and −16.7 ± 2.1 mV (n = 9) respectively and no significant differences exist among them. This result indicates that the mutations introduced at the amino terminal do not affect the voltage-dependent activation of Kv1.4 channel.

4. Discussion

It is well known that N-type inactivation of voltage-gated ion channel (ShB or Kv1.4) occurs through hydrophobic interaction between the amino-terminal inactivation ball and the channel's inner pore [2,7,15,20]. Despite that, several studies have demonstrated the important roles of electrostatic interactions in this type of inactivation process [2,7,21]. For example, the positively-charged hydrophilic domain of inactivation ball has been proved to accelerate the inactivation process of ShB channel [2]. Deleting charged residues of this region or neutralizing them slowed the inactivation of ShB channel induced by the inactivation domain or synthetic peptide [2,16]. Similarly, deletion of the positively-charged hydrophilic region (residues 26–37) of the Kv1.4 channel greatly decelerated its inactivation rate [7,13]. According to the crystal structure of several mammalian voltage-gated ion channels, the inactivation ball has to cross through the side portal between T1-S1 linkers to exert its function [22–24]. The long-range electrostatic interaction between the positively-charged inactivation ball and the T1-S1 linker region of the Kv1.4 channel has been identified with mutagenesis method [18]. Moreover, we have studied the electrostatic interaction between two oppositely-charged domains (positively-charged residues 87-98 and negatively-charged residues 123-137) and proved that the electrostatic attraction between them accelerates the inactivation process of Kv1.4 channel [25]. All these investigations suggest that the electrostatic interactions play an important

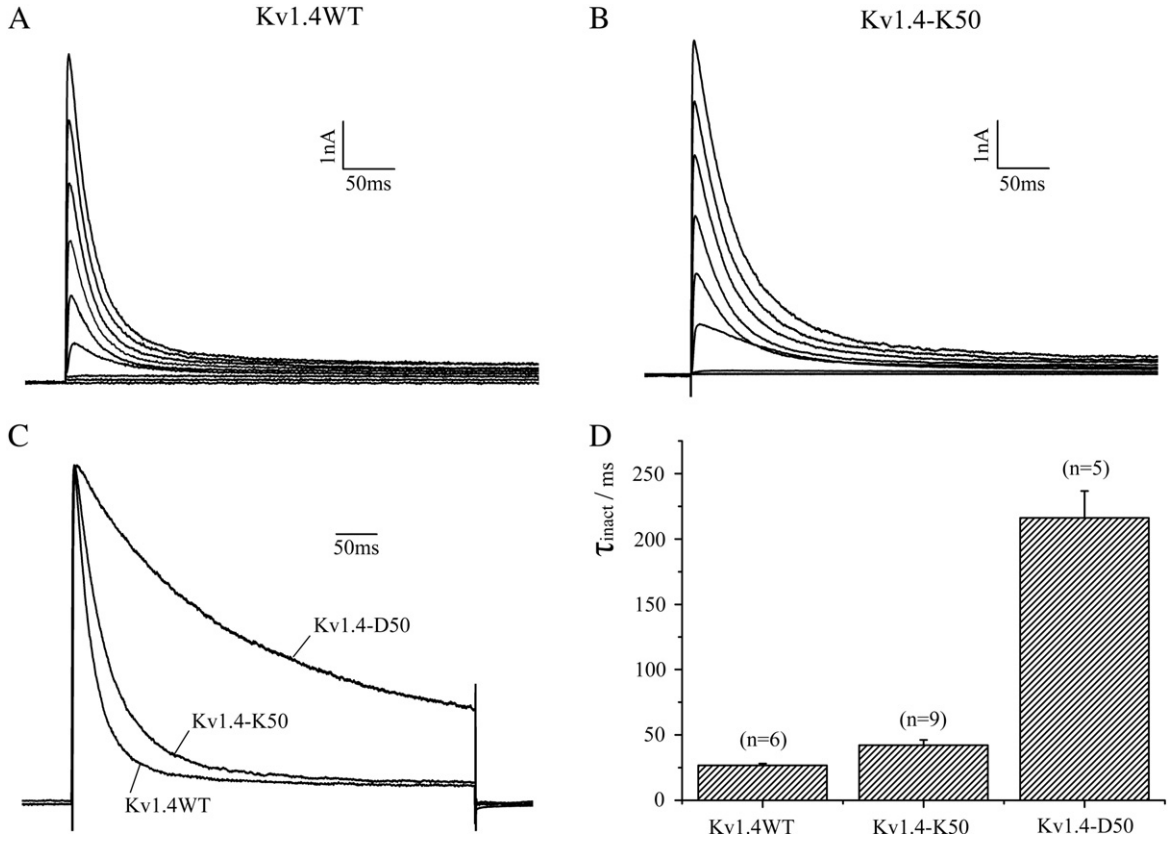

Fig. 4. Effect of K-peptide inserting mutation after residue 50A on inactivation of Kv1.4 channel. (A-B) Representative whole-cell current traces for Kv1.4WT and Kv1.4-K50. (C)The normalized and superimposed current traces for Kv1.4WT, Kv1.4-K50 and Kv1.4-D50 at +70 mV respectively. (D) Comparison of inactivation time constants (τ_{inact}) at +70 mV for Kv1.4WT, Kv1.4-K50 and Kv1.4-D50. The number of cells tested is given in the parentheses.

role in the N-type inactivation process of voltage-gated ion channels. In this study, we inserted positively- or negatively-charged short peptides at amino terminal of the Kv1.4 channel and examined their effects on the inactivation process. The results that inserting a negatively-charged short peptide decelerates the inactivation process far more severely compared with inserting a short peptide carrying positive charges and that mutant of inserting myc-peptide inactivates faster than mutant of inserting D-peptide both indicate the role of charged residues of NH_2-terminal in the inactivation process of Kv1.4 channel.

C-myc tag is a 10-amino-acid polypeptide protein tag (EQKLISEEDL) derived from the c-myc gene and can be added to a protein using recombinant DNA technology. This short peptide is chosen as an inserting peptide in this study because of the 3 net negative charges it carrying. It is presumable that inserting negatively-charged myc-peptide will decelerate the inactivation rate of Kv1.4 channel through electrostatic repulsion between myc-peptide and acidic T1-S1 linker region, since the positive charges on inactivation domain have been demonstrated to accelerate the inactivation process by its electrostatic attractions with the negatively charged T1-S1 linker [7,18]. In this study we choose 3 positions as the inserting sites which are all at unstructured and flexible region according to NMR-derived solution structure of NH_2-terminal of Kv1.4 channel [12]. The results that all of the 3 mutants exhibit significantly slower inactivation rates compared with Kv1.4WT indicate the decelerating role of electrostatic repulsion between the inserting myc-peptide and T1-S1 linker region. In our previous paper, we proposed that the electrostatic interaction between two oppositely-charged segments (segment A and segment B) makes the inactivation domain of Kv1.4 channel upward [25] and this structure further makes the NH_2-terminal of inactivation domain get closer to its electrostatic interacting site (T1-S1 linker) and enhances the electrostatic interaction of NH_2-terminal with T1-S1 linker region. Also because of the upward direction of NH_2-terminal caused by electrostatic attraction between segment A and segment B, the inserting peptide, which is nearer to the NH_2-terminal, has a larger electrostatic interaction with T1-S1 linker region. Considering this, the result that myc-peptide or D-peptide inserting mutant with inserting site nearer to the extreme NH_2-terminal decelerates the inactivation rate of Kv1.4 channel to a greater extent is understandable. For all the myc-peptide inserting mutants and wide type Kv1.4 channel, the inactivation rate constants do not show apparently voltage dependence at potentials where activation is complete, which reflects the voltage insensitivity of N-terminal binding at positive potentials. This result is consistent with the inactivation model in the recent study of Bett et al., which presumed that N-type inactivation of Kv1.4 channel can only occurs through the open state [26].

Although carrying the same number of net negative charges, the mutant of inserting myc-peptide inactivates more rapidly than the corresponding mutant inserting D-peptide. Comparing with D-peptide, myc-peptide sequence is 3 AA longer and carries two more charged amino acids—E and K at the NH_2-terminal. Perhaps the positively charged K attenuates the electrostatic field strength caused by negative charges of myc-peptide or the negative charges are partly buried into the secondary or tertiary structure formed by myc-peptide and/or its neighboring amino acid sequence of Kv1.4 channel, which finally induced the smaller decelerating effect on the inactivation rate caused by myc-peptide. Even so, what induced such different effects caused by myc-peptide and D-peptide can not be obtained from this study.

To investigate the role of charges-carrying peptide introduced outside further, we inserted a positively-charged K-peptide (SGKRKSG) behind the position 50A. Considering the possible electrostatic attraction with negatively-charged T1-S1 linker region introduced by positive charges of K-peptide, this mutation has been assumed to accelerate the inactivation rate of Kv1.4. Unexpectedly, the mutant of inserting K-peptide

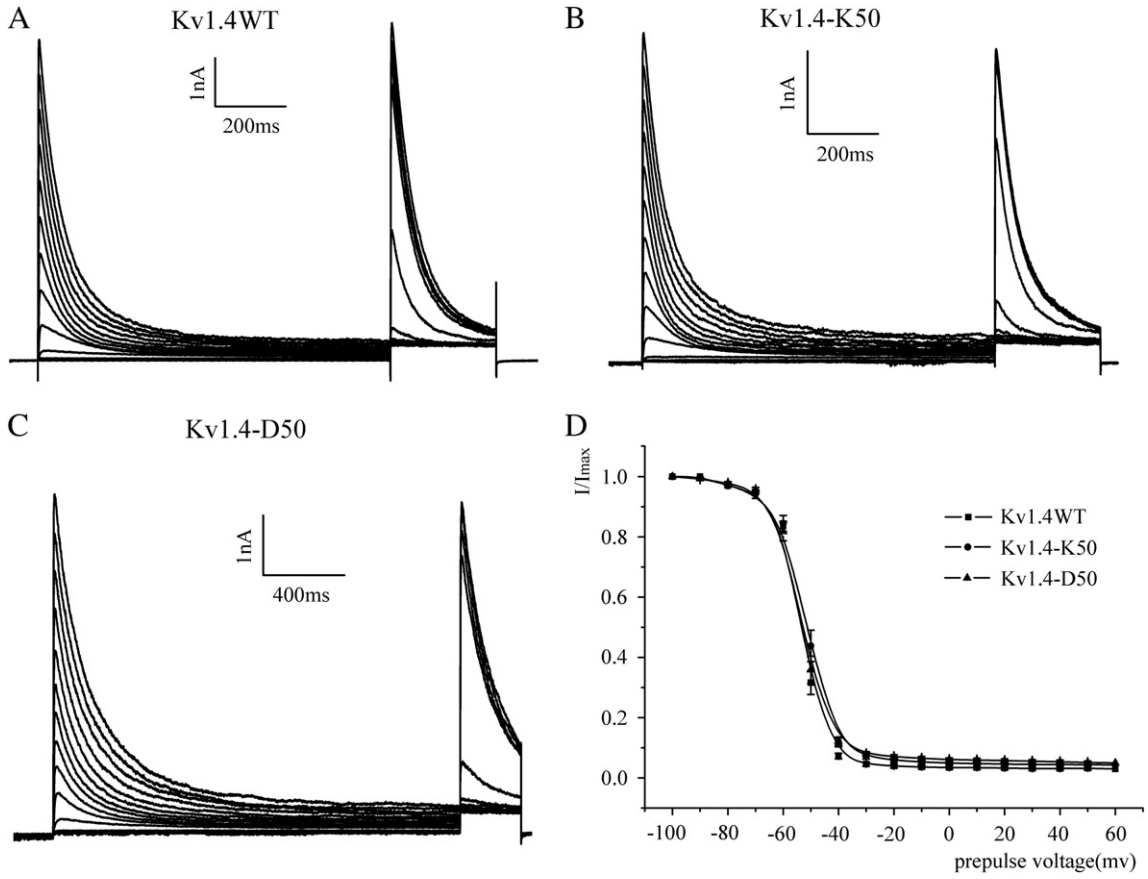

Fig. 5. Voltage-dependence of inactivation. (A–C) Currents elicited upon the two pulse protocol for Kv1.4WT, Kv1.4-K50 and Kv1.4-D50 respectively. (E) The normalized peak currents of test pulse as a function of pre pulse potentials. The voltage-dependent inactivation curve was fitted with a Boltzmann function ($I/Imax = 1/[1+\exp(V-V_{1/2})/k]$) to obtain the half inactivation potential ($V_{1/2}$).

exhibited a slower inactivation rate compared with Kv1.4WT. Besides the electrostatic interactions between inactivation domain and T1-S1 linker, there are also electrostatic attractions between another two charged segments (positively-charged segment A: 83-98 and negatively-charged segment B: 123-137) which have been proved to accelerate the inactivation of Kv1.4 channel in our previous study [25]. So, besides electrostatic attraction with acidic T1-S1 linker region, the inserted K peptide may also has electrostatic interactions with segment A and segment B, which finally influence its effect on inactivation of Kv1.4 channel. On the other hand, according to Wissmann's study, residues 40-50 following ID1 domain (residues 1-38) constitute a second inactivation domain (ID2), which, as a docking domain, promotes inactivation of Kv1.4 channel by attaching ID1 near the mouth of the channel pore [12]. Inserting K-peptide after residue 50 might also affect the role of ID2 as a docking domain and thus decelerates the inactivation of Kv1.4 channel.

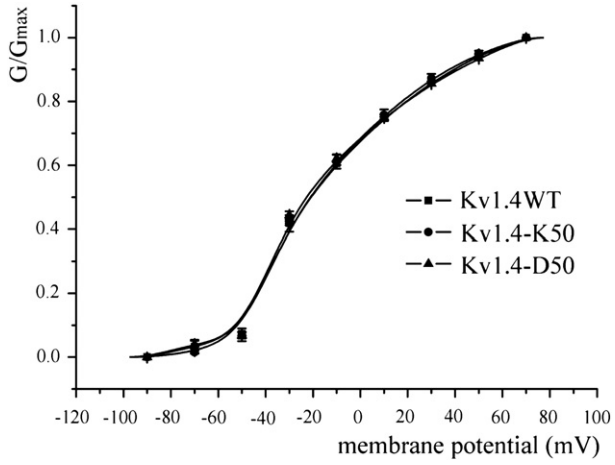

Fig. 6. Voltage-dependence of activation for Kv1.4WT, Kv1.4-K50 and Kv1.4-D50 respectively. Peak whole cell conductance (G) at each test potential (V) was calculated from $G = I_{peak}/(V-E_K)$, in which I_{peak} is the peak current at test potential and E_K is the equilibrium potential for K^+ calculated by Nernst function. G values were normalized for maximum conductance and plotted as a function of test membrane potential. The half activation potential ($V_{1/2}$) was obtained by fitting the curves of voltage-dependence of activation with the Boltamann equation $G/Gmax = 1/[1+\exp(V_{1/2}-V)/k]$, in which k is the slope factor for the activation curve.

Contrast to the large difference of inactivation rates, the voltage dependence of inactivation and activation are not changed significantly by these amino-terminal mutations, suggesting that the mutations done at the amino terminal do not disturb the structure of the transmembrane part of Kv1.4 channel.

This study investigated the effect of inserting negatively- or positively-charged short peptides on inactivation rate of Kv1.4 channel. Besides the present study, our two other previous studies have also investigated the effect of electrostatic interactions on inactivation of Kv1.4 channel, both of which have been mentioned above. One study investigated the electrostatic attraction between two appositively charged segments (Segment A: residues 83-98, Segment B: residues 123-137) of chain region of Kv1.4, the other study identified the electrostatic interaction between inactivation ball and T1-S1 linker region [18,25]. Based on these studies we could illustrate the N-type inactivation process of Kv1.4 channel influenced by electrostatic interactions. Firstly, electrostatic interaction between segment A and segment B of the chain region limits the movement of inactivation ball and shortens its passage for inactivating the

channel. Secondly, the electrostatic attraction between inactivation ball and T1-S1 linker region directs the motion of the inactivation ball towards its binding site and accelerates the inactivation process of Kv1.4 channel. The results of the present study do not propose new viewpoints about the inactivation of Kv1.4 channel but further identify the role of electrostatic interaction between inactivation ball and T1-S1 linker by inserting charged peptides on the inactivation domain of Kv1.4 channel. Liebovitch et al. have construct a model which quantitatively described the distribution of walk duration ($f(t)$) using analytical and numerical methods [27]. In their model, electrostatic interaction between the ball and its binding site only weakly affected the dependence of the rate constant on the chain length. In contrast, the influences of electrostatic interactions in the inactivation process of Kv1.4 channel seem larger in our studies. Besides hydrophobic and electrostatic interactions, N-type inactivation might be affected by many other factors. For example, the COOH-terminal might also participate in the inactivation process by interacting with the NH_2-teriminal [28,29]. In fact, the rapid N-type inactivation process is so elaborate that it still has not been totally understood despite so many investigations.

Acknowledgements

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